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Dietary and Physiological Contributions to the Relationship between Diet, Bone Collagen, and Structural Carbonate δ13C Values

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Abstract
Numerous models have been proposed to explain and predict the relationship between diet, bone collagen, and structural carbonate $\delta^{13}C$ values. Within these models, many internal and external factors are implicated in generating the observed variation in $\delta^{13}C$ values, such as trophic level, dietary protein source, digestive physiology, tissue growth and remodeling, and post-mortem chemical alteration of bone collagen and bone mineral. The current understanding of the relationship between the isotopic chemistry of bone and diet hinges on the observation that bone collagen and structural carbonate fractionate differentially from diet due to underlying metabolic differences. The stable carbon isotopic composition of bone collagen is shown to strongly reflect the isotopic composition of dietary protein. In contrast, the stable carbon isotopic composition of structural carbonate within bone mineral is representative of the isotopic composition of total diet. The spacing between the $\delta^{13}C$ values of bone collagen and structural carbonate is often used as a measure for understanding variation in the isotopic composition of dietary protein relative to total diet. However, the complexity of the diet-tissue relationship often provides limitations and challenges to paleodietary reconstruction using stable isotopic analysis. This paper explores some of the dietary and physiological factors producing and affecting the relationship between diet, bone collagen, and structural carbonate $\delta^{13}C$ values.

Keywords
stable isotope analysis, bone collagen, structural carbonate, collagen-carbonate spacing

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Introduction

Stable carbon isotope signatures of bones and teeth are commonly used by archaeologists to reconstruct the diets of past populations. The relative dietary contributions of C_3, C_4, and marine carbon signals in the diets of humans and other animals can be determined through the analysis of stable carbon isotope ratios in consumer tissues. For instance, stable carbon isotopic analyses have enabled archaeologists to document the rise and spread of maize agriculture in the Americas (Bender et al. 1981; Katzenberg et al. 1995; Schwarcz et al. 1985; van der Merwe and Vogel 1978). The most common tissue components used in stable carbon isotopic analyses of archaeological bones and teeth are type I collagen and structural carbonate (Ambrose 1993). Type I collagen is the main constituent of the organic phase of bones and dentin. The organic phase comprises approximately 30% of the mass of bones and 20% of the mass of dentin by some estimates (Davis 1987; Linde and Goldberg 1993). Structural carbonate is suspended within the crystal lattice of hydroxyapatite – a type of calcium phosphate that forms the inorganic phase of bones, dentin, and enamel. The inorganic phase accounts for approximately 70% of the mass of bones, 80% of the mass of dentin, and 99% of the mass of enamel (Davis 1987; Linde and Goldberg 1993).

The stable carbon isotopic compositions of bone collagen and structural carbonate reflect the diets of individuals in slightly different ways because of underlying differences in their respective syntheses. The carbon atoms used to synthesize bone collagen and structural carbonate are transferred through different metabolic processes. The stable carbon isotopic compositions of bone collagen are shown to derive a large proportion of their carbon atoms directly from dietary protein (Ambrose and Norr 1993; Jim et al. 2004; Kellner and Schoeninger 2007; Schwarcz 2000; Tieszen and Fagre 1993). Collagenous carbon is strongly representative of the protein component of total diet. In comparison, the carbon in structural carbonate is derived from bicarbonate in the blood, which is representative of all macronutrients within the total diet (Ambrose and Norr 1993; Tieszen and Fagre 1993). The relationship between the $\delta^{13}C$ values of collagen and structural carbonate, or the collagen-carbonate spacing ($\Delta_{co\text{-}ca}$), has been demonstrated to elucidate further information about dietary protein sources (Ambrose et al. 1997; Harrison and Katzenberg 2003). Collagen-carbonate spacing reveals variability in the isotopic compositions of dietary protein relative to total diet. This paper explores and reviews some of the dietary and physiological factors that influence the relationship between diet, bone collagen, and structural carbonate $\delta^{13}C$ values.

Bone Collagen and Structural Carbonate Synthesis

In order to understand how stable carbon isotopes are represented in bone collagen and structural carbonate, it is necessary to understand some elements of the structure and synthesis of the organic and inorganic phases of bone. Dietary macronutrients such as proteins, carbohydrates, and lipids, are digested by the body into constituent parts. In mammals, proteins are hydrolyzed into amino acids, carbohydrates are digested into sugars, and lipids are hydrolyzed into glycerol and fatty acids (Schwarcz 2000). Over 90% of

ingested carbon atoms are expired through the lungs as CO_2, but some will be used in the anabolism of tissues, including bone (Hedges 2003; Schwarcz 2000).

Collagens represent a group of molecules that are either fibrillar or non-fibrillar in structure. Fibrillar collagens, such as types I and II, are the main structural proteins in the extra-cellular matrix of bone and hyaline cartilage, respectively (von der Mark 2006). Bone collagen molecules are secreted into extra-cellular space as procollagen by osteoblasts, where they are cleaved by specific collagenases and aggregate into heterofibrils composed of type I and type V collagens (Rossert and de Crombrugghe 2002). Type I and type V collagens comprise approximately 90% and 5% of the non-mineralized phase of bones, respectively (Niyibizi and Eyre 1989; von der Mark 2006). Fibrillar collagen molecules are mainly composed of glycine, proline, and hydroxyproline repeats (Rossert and de Crombrugghe 2002). The amino acids in collagen molecules can be derived from the hydrolysis of dietary proteins, through *de novo* synthesis from other amino acid precursors, or through amino acid recycling during normal tissue turnover (Schwarcz 2000). Ambrose et al. (1997) argue that a minimum of 20% of dietary amino acids are selectively transferred, or "routed", to collagen synthesis because they cannot be synthesized within the body. The majority of amino acids in bone collagen are non-essential, meaning they can be synthesized *de novo* within the body if necessary. However, controlled dietary experiments show that there is a strong tendency toward the incorporation of non-essential amino acids directly from dietary sources, rather than through *de novo* synthesis (Ambrose and Norr 1993; Tieszen and Fagre 1993). Over 60% of the total amino acids in dietary protein are estimated to be routed to collagen synthesis (Ambrose and Norr 1993; Ambrose et al. 1997). As a result, the $\delta^{13}C$ values of collagen strongly reflect the stable carbon isotopic signatures of dietary protein sources.

While collagen follows a routing model of metabolism, structural carbonate in bone hydroxyapatite conforms to a linear mixing model (Ambrose and Norr 1993; Tieszen and Fagre 1993). Following the synthesis of collagen fibrils by osteoblasts, the fibrils self-assemble into a cross-linked meshwork called osteoid (Robins 2006). Non-collagenous proteins bind to specific sites at the end-to-end collagen fibrils, acting as nuclei for hydroxyapatite precipitation (Robey 2008). Hydroxyapatite precipitates form these nuclei within osteoid, eventually forming bone. Structural carbonate (CO_3) within the crystal lattice of bone mineral is an impurity that replaces the phosphate groups (PO_4) of hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$) at specific sites (Wright and Schwarcz 1996). Structural carbonate is synthesized using the bulk metabolic carbon pool and derives its carbon from blood plasma CO_2, which is representative of all dietary constituents (Hedges 2003; Schwarcz 2000; Tieszen and Fagre 1993). There is no selective transfer of dietary macronutrients during structural carbonate synthesis. The isotopic composition of the total diet is in isotopic equilibrium with respired CO_2 (Tieszen and Fagre 1993). Respired CO_2 is in isotopic equilibrium with blood bicarbonate. Lastly, blood bicarbonate is in isotopic equilibrium with structural carbonate in bone. As a result, the $\delta^{13}C$ values of structural carbonate represent the average weighted $\delta^{13}C$ value of all dietary macronutrients (Hedges 2003; Schwarcz 2000). The average weighted $\delta^{13}C$ value ($\delta^{13}C_{diet}$) refers to the average $\delta^{13}C$ value of all individual dietary constituents (e.g. aquatic plants, grains, fruits, or meat) weighted by their relative dietary importance. In other words, the $\delta^{13}C$ values

of structural carbonate accurately reflect the $\delta^{13}C$ values of total diet.

Past Interpretations of Collagen-Carbonate Spacing in Fossil and Contemporary Bone

Numerous models have been proposed to explain and predict the relationship between the $\delta^{13}C$ values of diet, bone collagen, and structural carbonate. Sullivan and Krueger (1981) first presented observations on collagen-carbonate spacing, showing that $\Delta_{co\text{-}ca}$ is invariably about 8‰ across a variety of modern and fossil species. Diet-collagen spacing ($\Delta_{diet\text{-}co}$) was consistently observed to be +5‰ and structural carbonate in bone hydroxyapatite was consistently fractionated from dietary $\delta^{13}C$ values by +13‰ across species. In response, Schoeninger and DeNiro (1982, 1983) argued that $\Delta_{co\text{-}ca}$ was not a fixed amount, citing isotopic analyses in which the $\Delta_{co\text{-}ca}$ is highly variable. Instead, they suggested that collagen-carbonate spacing is a function of post-mortem chemical alteration of structural carbonates in bone. However, Shoeninger and DeNiro fail to explain the variation in collagen-carbonate spacing observed in modern samples. Post-mortem chemical alteration can significantly influence the $\delta^{13}C$ values of bone collagen and structural carbonate, although there is still an underlying variability in $\Delta_{co\text{-}ca}$ that is attributable in part to diet and physiology.

Krueger and Sullivan (1984) proposed that variation in collagen-carbonate spacing is a function of trophic level that manifests in predictable ways. According to their model, differing proportions of proteins, carbohydrates, and lipids in the diets of carnivores, omnivores, and herbivores produce variable $\Delta_{co\text{-}ca}$. In this model, the $\delta^{13}C$ values of collagen are considered representative of the protein component of the diet, whereas the $\delta^{13}C$ values of structural carbonate are considered representative of the energy component of the diet, specifically carbohydrates, lipids, and proteins not used in protein synthesis. Krueger and Sullivan (1984) maintain that herbivores derive protein from plant proteins and the catabolism of plant carbohydrates. Herbivores derive energy from dietary carbohydrates, lipids, and proteins not used in protein synthesis. Carnivores derive protein directly from dietary protein sources and energy primarily from dietary lipids and proteins. Lipids are depleted in ^{13}C relative to other tissues due to isotopic fractionation during lipogenesis (DeNiro and Epstein 1977). Krueger and Sullivan (1984) posit that a higher proportion of lipids used in energy metabolism results in more negative $\delta^{13}C$ values of structural carbonate, while the $\delta^{13}C$ values of bone collagen are unaffected. The shift toward more negative $\delta^{13}C_{ca}$ values relative to $\delta^{13}C_{co}$ produces smaller collagen-carbonate spacing in carnivores.

Similarly, Lee-Thorp, Sealy, and van der Merwe (1989) report that the average $\Delta_{co\text{-}ca}$ of herbivores is 6.8 ± 1.35‰, 4.3 ± 1.0‰ for carnivores, and 5.2 ± 0.8‰ for omnivores within a sample of wild South African fauna. Based on this observation, Lee-Thorp, Sealy, and van der Merwe (1989) argue that collagen-carbonate spacing is a measure of the importance of meat in the diets of omnivores. This index may be applied to archaeological human populations in order to determine the relative importance of animal protein in the diets of past populations. When Lee-Thorp, Sealy, and van der Merwe (1989) apply this index to an archaeological human population from South Africa, the average $\Delta_{co\text{-}ca}$ was found to be 2.6 ± 1.0‰. An average $\Delta_{co\text{-}ca}$ of 2.6 ±1.0‰ indicates that this human group was consuming more meat than strictly carnivorous animals, which does not conform to the expectations of their model. Controlled dietary studies have since demonstrated that variability in $\Delta_{co\text{-}ca}$ arises

from variation between the $\delta^{13}C$ values of dietary protein and total diet (Ambrose and Norr 1993; Tieszen and Fagre 1993). In opposition to past models, the $\delta^{13}C$ values of structural carbonate are shown to fractionate from $\delta^{13}C_{diet}$ by a relatively constant amount, while variation in the $\delta^{13}C$ values of collagen account for increases or decreases in collagen-carbonate spacing.

Contemporary Approaches to the Study of Collagen-Carbonate Spacing

The current understanding of variation in collagen-carbonate spacing is that routing of dietary protein to bone collagen anabolism increases or decreases $\Delta_{co\text{-}ca}$ based on the $\delta^{13}C$ value of dietary protein relative to $\delta^{13}C_{diet}$ in chemically unaltered bone. Controlled dietary experiments show that diet-carbonate spacing in mammals with mono-and non-mono-isotopic diets is on average 9.5‰ (Ambrose and Norr 1993, 9.4‰; DeNiro and Epstein 1978, 9.6‰; Jim et al. 2004, 9.5‰; Kellner and Schoeninger 2007, 9.7‰). Diet-collagen spacing is approximately 5‰ in animals with monoisotopic diets or when the $\delta^{13}C$ value of dietary protein is similar to $\delta^{13}C_{diet}$ (Ambrose and Norr 1993; van der Merwe and Vogel 1978; Vogel 1978). Collagen-carbonate spacing that arises purely from metabolic differences in collagen and structural carbonate synthesis should theoretically equal 4.5‰ because $\Delta_{co\text{-}ca}$ is equal to the difference between diet-carbonate fractionation (9.5‰) and diet-collagen fractionation (5‰). In animals of similar age, digestive physiology, and nutritional status, deviations from the theoretical average value of $\Delta_{co\text{-}ca}$ can occur when dietary protein is significantly depleted or enriched in ^{13}C relative to $\delta^{13}C_{diet}$. This phenomenon accounts for the observed collagen-carbonate spacing in the archaeological population from South Africa examined by Lee-Thorp, Sealy, and van der Merwe (1989). The population lived along the South African coast and consumed primarily marine protein supplemented by C_3 carbohydrates and lipids. The $\delta^{13}C$ values of bone collagen from this population reflect a heavy reliance on marine protein, which is enriched in ^{13}C, whereas the $\delta^{13}C$ values of structural carbonate reflect the average $\delta^{13}C$ value of total diet. The theoretical spacing of 4.5‰ between the $\delta^{13}C$ values of collagen and structural carbonate is reduced to 2.6 ± 1.0‰ by the shift to less negative $\delta^{13}C$ values of collagen due to the ^{13}C-enriched carbon signal from marine protein. In general, for animals with similar physiology, diets consisting of C_3 protein and C_4/marine non-protein produce collagen-carbonate spacing greater than 4.5‰, whereas diets consisting of C_4/marine protein and C_3 non-protein produce $\Delta_{co\text{-}ca}$ lower than 4.5‰ (Ambrose et al. 1997).

Collagen-carbonate spacing reveals information about the isotopic composition of dietary protein ingested by humans and other animals, although there are numerous other factors aside from the isotopic composition of dietary protein relative to total diet that can affect $\Delta_{co\text{-}ca}$. Digestive physiology, growth, nutritional status, and normal tissue turnover may impact the relationship between diet, bone collagen, and structural carbonate $\delta^{13}C$ values during life. For example, the proximate cause of greater $\Delta_{co\text{-}ca}$ on average in many herbivores is argued to be attributable to underlying differences in digestive physiology between ruminants and non-ruminants. Ruminant herbivores with fore- or hind-gut fermentation show greater diet-carbonate spacing, which is potentially caused by methanogenesis during fermentation (Hedges 2003; Hedges and van Klinken 2000). With respect to growth, Schwarcz (2000) argues that changes in the isotopic equilibrium between $\delta^{13}C_{diet}$ and $\delta^{13}C_{ca}$ arising from tissue growth can alter the

relationship between diet, collagen, and structural carbonate $\delta^{13}C$ values. Some ingested carbon may be immediately channelled into tissue growth and is not balanced by catabolic oxidative processes, which could preferentially deplete the carbon pool of specific isotopes. Considerable work on the stable nitrogen isotopic compositions of growing bones demonstrates that rapid growth can influence $\delta^{15}N$ values (see Waters-Rist and Katzenberg 2010), but the effects of growth on stable carbon isotope ratios are currently poorly understood.

Similarly, during normal remodeling of adult bones, the hydrochloric acid secreted by osteoclasts may preferentially liberate molecules containing lighter carbon isotopes due to their weaker bond strength, producing *in vivo* changes to the stable isotopic composition of bone. The preferential incorporation of ^{15}N over ^{14}N is argued to arise during the processing of nitrogen for excretion (DeNiro and Epstein 1981). Amino acids containing ^{14}N are more easily deaminated than those containing ^{15}N due to the weaker bonds of ^{14}N isotopes, which results in the preferential breakdown of amino acids containing lighter isotopes (Macko et al. 1986). A similar process of preferential molecular breakdown may occur during bone resorption, but has yet to be empirically investigated – the impact of bone remodeling on the diet-tissue relationship is currently unclear.

Body size is unlikely to significantly contribute to the relationship between $\delta^{13}C$ values of diet, collagen, and structural carbonate as evidenced by Froehle, Kellner, and Shoeninger (2010). Froehle, Kellner, and Schoeninger (2010) present a model for estimating protein source when $\delta^{13}C_{diet}$ is unknown based on controlled dietary studies of mice (Ambrose and Norr 1993; Tieszen and Fagre 1993), rats (Jim et al. 2004), and pigs (Hare et al. 1991; Howland et al. 2003; Warinner and Tuross 2009). There is significant uniformity in the relationship between collagen and structural carbonate $\delta^{13}C$ values, which suggests that body size does not heavily factor into this relationship when digestive physiology and maturity are similar. Ultimately, the relationship between the $\delta^{13}C$ values of diet, bone collagen, and structural carbonate is not limited to dietary and physiological factors. Post-mortem chemical alteration is often implicated as a contributing factor in this relationship (Schwarcz 1991; Wright and Schwarcz 1996), but will not be discussed here.

Conclusions

Since the first models proposed to quantify the diet-tissue relationship using $\delta^{13}C$ values in humans and other animals, there has been increasing integration of principles of biochemistry and mammalian physiology into isotopic analyses. In the past, stable isotope scientists have often referred to the human body as a "black box", wherein dietary macronutrients are processed, rearranged, and transformed, generating a quantifiable yet poorly understood chemical signature. Efforts in understanding the varying metabolic pathways that dietary carbon may enter have been instrumental in demonstrating the relationship between the isotopic signatures of dietary macronutrients and bodily tissues. For instance, the discovery of the routing of dietary protein toward collagen anabolism was a major stride in stable isotope anthropology, whereas it had previously been assumed to follow a linear mixing model. With continued efforts, our understanding of the relationship between the $\delta^{13}C$ values of diet, bone collagen, and structural carbonate is certain to be enhanced. Further research on the role of digestive physiology, growth and development, nutritional status, and tissue remodeling in the diet-tissue relationship is

required to obtain a more comprehensive perspective on diet-tissue interaction and to make our interpretations as transparent as possible. There is an increasing need for interdisciplinary consultation between stable isotope anthropologists, biogeochemists, mammalian physiologists, and cell biologists in order to progress and transform the science of paleodietary reconstruction using stable isotopic analysis. Future interdisciplinary collaboration between these disciplines will undoubtedly contribute to a greater understanding of our collective past simply by understanding the present.

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